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Recombinant Interleukin-1α and Recombinant Tumor Necrosis Factor α Synergize In Vivo To Induce Early Endotoxin Tolerance and Associated Hematopoietic Changes

STEFANIE N. VOGEL,[1]* ERIC N. KAUFMAN,[1] MICHELE D. TATE,[1] AND RUTH NETA[2]

Department of Microbiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road,[1] and Department of Experimental Hematology, Armed Forces Radiobiology Research Institute,[2] Bethesda, Maryland 20814

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Endotoxin, the lipopolysaccharide (LPS) derived from gram-negative bacteria, invokes a wide range of responses in susceptible hosts. It is known that virtually all responses to LPS are mediated by the action of macrophage-derived cytokines (such as interleukin-1 [IL-1], tumor necrosis factor [TNF], and others) which are produced principally by macrophages and maximally within several hours of LPS administration. One manifestation of LPS administration which is not well understood is the phenomenon of "early endotoxin tolerance." In response to a single sublethal injection of LPS, experimental animals become refractory to challenge with a homologous or heterologous LPS preparation 3 to 4 days later. Animals rendered tolerant exhibit mitigated toxicity and a reduced capacity to produce circulating cytokines (i.e., colony-stimulating factor or interferon) in response to the challenge LPS injection. Previous studies have also shown that this state of transient, acquired hyporesponsiveness to LPS is accompanied by a marked increase in the size of cells in the bone marrow which are enriched in numbers of macrophage progenitors. In this study, we examined the capacity of recombinant IL-1 or recombinant TNF or both to induce early endotoxin tolerance and its associated hematopoietic changes. Neither cytokine alone was able to mimic LPS for induction of tolerance. Combined administration of recombinant IL-1 and recombinant TNF doses which were not toxic when administered individually led to synergistic toxicity (as assessed by death or weight loss). However, within a nontoxic range, the two cytokines synergized to induce a significant reduction in the capacity to produce colony-stimulating factor in response to LPS, as well as the characteristic increase in bone marrow cell size and macrophage progenitors shown previously to be associated with LPS-induced tolerance. Keywords: Toxic tolerance, Immunity, Reprints (AW)

Endotoxin, the lipopolysaccharide (LPS) component of gram-negative bacterial outer membranes, induces in vivo many of the pathophysiologic changes associated with systemic gram-negative bacterial infection (reviewed by S. N. Vogel and M. M. Hogan, *in* J. J. Oppenheim and E. Sherach, ed., *Immunophysiology: Role of Cells and Cytokines in Immunity and Inflammation*, in press). Among these are fever, hypoglycemia, hypotension, shock, and even death. Several lines of evidence support the hypothesis that the macrophage is the principal cellular mediator of endotoxicity and, more specifically, that LPS-induced, macrophage-derived soluble factors are the direct mediators of endotoxic phenomena. First, LPS-stimulated macrophages produce in vitro many of the same soluble factors which circulate in the serum following in vivo administration of LPS. These include interleukin-1 (IL-1), tumor necrosis factor (TNF; also referred to as cachectin), interferon, colony-stimulating factor (CSF), and prostaglandins of the E series. When purified and injected into experimental animals in the absence of LPS, many of these soluble factors have been shown to induce one or more of the effects of in vivo LPS treatment. Agents which increase macrophage activation greatly increase endotoxin sensitivity in vivo, even in the genetically LPS-hyporesponsive C3H/HeJ mouse strain (23, 27). Increased sensitivity to LPS correlates well with quantifiable increases in levels of circulating LPS-induced factors (5, 13, 24, 27, 32). In studies in which antisera against specific cytokines have been administered prior to or simultaneously with LPS, many of its biologic effects have been mitigated. For instance, anti-IL-1 serum has been shown to block LPS-induced fever (8). Similarly, injection of anti-TNF antibodies afforded significant protection against lethal challenge with LPS (4). Lastly, in mouse strains that bear the defective allele for LPS responsiveness, *Lps*^{d}, protein-free preparations of LPS fail to elicit the production of macrophage-derived factors, either in vivo or in vitro (reviewed by Vogel and Hogan [in press]).

A single injection of LPS results in the appearance of a temporal hierarchy of factors within the serum (reviewed by Vogel and Hogan [in press]). The first group of cytokines (i.e., IL-1, TNF, and interferon) appears maximally within 2 h of injection, and they have been referred to as "early acute-phase reactants." By 4 to 8 h postinjection, circulating levels of these factors are greatly decreased. Production of granulocyte-macrophage CSF activity is somewhat delayed, with peak activity occurring at 4 to 6 h following LPS and declining to basal levels by 24 h. A third group of soluble factors which appears maximally in the serum 18 to 24 h postinjection has been collectively referred to as "late acute-phase reactants" and includes C-reactive protein, serum amyloid A, fibrinogen, and others. This last group of factors is produced primarily by hepatocytes and is induced by the action of early acute-phase reactants on these cells.

For many years, it has been recognized that initial sublethal exposure to LPS renders experimental animals refractory to a subsequent challenge with homologous or heterologous LPS several days later. This effect was referred to as "early-phase endotoxin tolerance" (reviewed in reference 9). Until recently, very little was known about the mechanisms which underlie this phenomenon. Early studies dem-

* Corresponding author.

onstrated that macrophages derived from mice which had received a tolerance-inducing injection of LPS failed to respond to subsequent LPS challenge in vitro to produce endogenous pyrogen or prostaglandins (7, 21). In this sense, macrophages from mice rendered tolerant are phenotypically similar to macrophages rendered refractory to LPS by pharmacological means (e.g., by treatment with glucocorticoids; 3, 28). Williams et al. (31) demonstrated that a splenic adherent cell population was necessary for abrogation of early-phase endotoxin tolerance in transfer experiments, supporting the cellular nature of this phenomenon. In studies performed in this laboratory, it was demonstrated that early-phase endotoxin tolerance is associated with alterations in bone marrow-derived macrophage precursor pools (10, 11). Specifically, cell-sizing profiles of bone marrow cells from mice rendered tolerant showed enrichment for a population of cells significantly larger than control bone marrow populations, and by density gradient sedimentation it was shown that the denser population of cells contained increased numbers of macrophage progenitors. The induction, maintenance, and loss of these hematopoietic changes coincided temporally with the acquisition, maintenance, and loss of the tolerant state. These changes were observed in a variety of outbred and inbred mouse strains, including those with defects or deficiencies within certain lymphoid cell subsets. For example, early-phase endotoxin tolerance, as well as the associated hematopoietic changes, were observed in athymic (nude), B-cell-deficient (*xid*), and splenectomized mice (12). As an additional control, LPS-hyporesponsive C3H/HeJ mice did not exhibit any of the hematopoietic alterations observed in fully LPS-responsive mice rendered tolerant by injection of LPS (11).

In this study, we tested the possibility that cytokines which are normally produced in response to a tolerance-inducing dose of LPS may mediate the induction of early endotoxin tolerance or the accompanying hematopoietic alterations or both. Our findings indicate that combined treatment of mice with recombinant IL-1α (rIL-1) and recombinant TNFα (rTNF) induces a significant, synergistic level of toxicity, but at sublethal-dose ranges they induce a significant degree of endotoxin tolerance, as well as changes in bone marrow cell-sizing profiles and enrichment for macrophage progenitors previously associated with the tolerant state.

MATERIALS AND METHODS

Mice. Female C57BL/6J mice 5 to 6 weeks old were purchased from Jackson Laboratory (Bar Harbor, Maine) and used within 2 weeks of their receipt. Mice were allowed access to food and acid water ad libitum.

Reagents. Protein-free LPS was prepared from *Escherichia coli* K235 by the phenol-water extraction method of McIntire et al. (14). Human rIL-1α (lot SM59) was the generous gift of Hoffmann-LaRoche Inc. (Nutley, N.J.) and possessed a specific activity of 5×10^6 U/mg. The activity of this material was verified frequently in a standard thymocyte comitogenic assay (25) throughout the course of this study. The concentration of contaminating LPS in this rIL-1 preparation was 1.5 ng/1.3×10^6 U. Human rTNFα (lots NP102 and NP200B) was the generous gift of Cetus Corporation (Emeryville, Calif.) and possessed a specific activity of approximately 2×10^7 U/mg. The activity of the rTNF was also verified in a standard actinomycin D-treated L929 fibroblast cytotoxicity assay (29). The concentration of LPS in the rTNF preparations was <0.03 ng/0.3 mg. All reagents were maintained at −20°C, either in lyophilized form or as intermediate stocks at high protein concentrations, before dilution in pyrogen-free saline just before injection.

Measurement of CSF activity in serum. Serum was tested for CSF activity in a bone marrow colony assay in semisolid agar as described previously (11). Briefly, serum was obtained from pooled blood collected from mice injected (as indicated) with saline, rIL-1, rTNF, or LPS. Serial dilutions of serum were made in six-well tissue culture plates (Costar, Cambridge, Mass.). Bone marrow cells were obtained from the femurs and tibias of C3H/HeJ mice and processed by density gradient centrifugation in lymphocyte separation medium (Litton Bionetics, Kensington, Md.). The cells were then collected from the gradient interface and diluted to a final concentration of 10^5 cells per ml in a mixture of tissue culture medium and molten agar. One milliliter of the cell suspension was added to each of the wells, which contained 0.2 ml of the serum dilution. The wells were mixed by swirling and allowed to solidify. Cultures were incubated at 37°C (6% CO_2) for 7 days, at which time bone marrow colonies (≥25 cells) were enumerated with an inverted microscope.

Determination of the number of macrophage progenitor cells in bone marrow. The number of macrophage progenitor cells was determined as described elsewhere (11). Briefly, mice from each experimental group were sacrificed, and the bone marrow cells were obtained from the femurs and tibias by flushing with serum-free medium. The bone marrow cells were centrifuged and suspended in tissue culture medium. Cell counts and cell-sizing profiles were obtained with a model ZM Coulter Counter and a C1000 Channelyzer (Coulter Electronics, Inc., Hialeah, Fla.) calibrated as directed by the manufacturer.

To determine the number of macrophage progenitors, a double-layer, semisolid agar colony assay was used as described previously (11). An excess of partially purified murine macrophage CSF (CSF-1) was incorporated into the bottom layer of the assay system. Bone marrow cells (5×10^4 or 1×10^5) were then suspended in a molten agar medium mixture and overlaid onto the CSF-1-containing layer. Colonies (≥50 cells) were enumerated after 10 days in culture, and the number of progenitors per 10^5 input bone marrow cells was calculated.

Injection schedule for induction of early endotoxin tolerance. The protocol for induction of early endotoxin tolerance was identical to that used in previous studies (10–12, 31). Briefly, mice were injected intraperitoneally with 25 μg of *E. coli* K235 LPS in a volume of 0.5 ml on day 0. Three days later, mice were challenged with 25 μg of LPS and bled 6 h later for measurement of CSF in serum. Controls included mice injected on days 0 and 3 with pyrogen-free saline and mice injected on day 0 with saline and on day 3 with LPS. Mice which received rIL-1 or rTNF or both were also injected on day 0 with the indicated concentrations of cytokines but challenged on day 3 with saline or LPS.

RESULTS

Toxicity of rIL-1 and rTNF when administered individually or in combination. In our previous studies, an early-phase endotoxin tolerance system was established in which injection of 25 to 50 μg of LPS (approximately 0.05 to 0.1 50% lethal dose) was shown to render mice significantly less responsive to an LPS challenge 3 to 4 days later. Since LPS has been shown to induce both IL-1 and TNF concurrently, and since both cytokines have been shown to mimic LPS

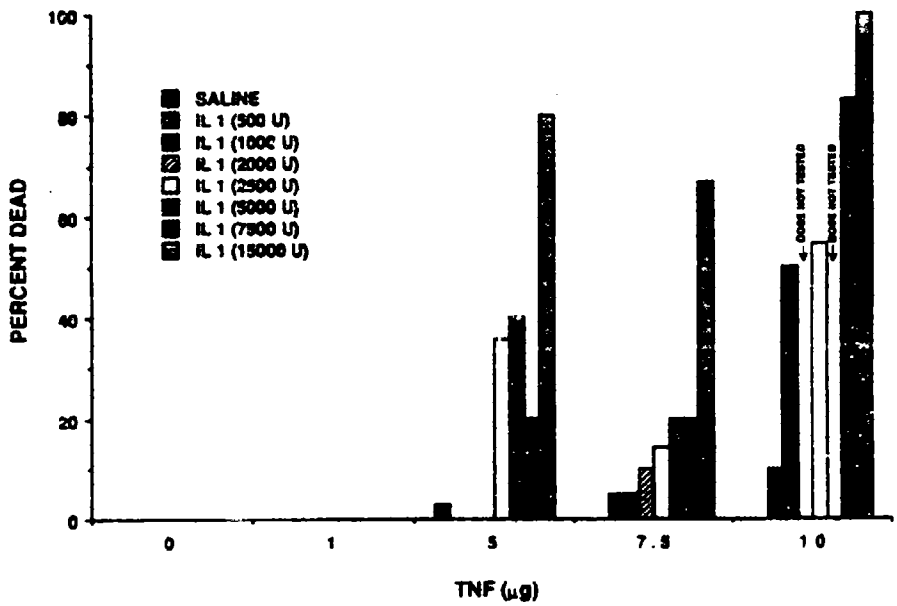

FIG. 1. Toxicity in response to injection with rIL-1 or rTNF or both. Mice were injected intraperitoneally with 0.5 ml of the indicated doses of rTNF (*x* axis) or rIL-1 (bar graph legends) or both, and the percentage of deaths (*y* axis) was scored over a 3-day period. Each point represents the total percentage of deaths observed for a given combination. Each combination was tested in 1 to 10 independent experiments in which six mice (average) per treatment per experiment were injected.

when administered in vivo (reviewed in reference 6 and by Vogel and Hogan [in press]), we sought to test the hypothesis that rIL-1 or rTNF or both could mediate induction of early endotoxin tolerance. Previous studies demonstrated that injection of high doses of either rIL-1 or rTNF resulted in production of levels of CSF comparable to those induced by 25 μg of LPS, i.e., the dose used for induction of early endotoxin tolerance (11). Therefore, the doses of rIL-1 and rTNF chosen for preliminary studies bracketed those used previously. Figure 1 illustrates the toxicity of these two cytokines over a very broad dose range when administered individually or in combination. The dose of rTNF injected is shown along the *x* axis, and the dose of rIL-1 injected is indicated by the bar graph legends. The percentage of deaths is plotted on the *y* axis. Injection of high doses of rTNF (up to 2×10^5 U per mouse; 10 μg) alone occasionally resulted in deaths (maximal toxicity never exceeded 3%). Injection of rIL-1 alone (up to 15,000 U per mouse; ~3 μg) was never lethal for mice. However, when ≥7.5 μg of rTNF was injected in combination with increasing doses of rIL-1, deaths (up to 100%) were observed in a dose-dependent fashion. In 10 separate experiments, 25 μg of LPS led to deaths in only 3.1% of the mice, consistent with our previous estimates of the 50% lethal dose for this particular endotoxin preparation (11, 26). On the basis of a recent report by Rothstein and Schreiber (22) which showed that rTNF synergized with LPS to induce deaths, we calculated the concentrations of contaminating LPS injected along with each cytokine. For the maximum dose of rTNF injected (i.e., 10 μg), <0.001 ng of LPS was injected. For the maximum dose of rIL-1 injected (i.e., 15,000 U), 0.017 ng of LPS was injected. Thus, even with the largest dose combination (i.e., 10 μg of rTNF and 15,000 U of rIL-1), only 0.018 ng of LPS was injected. This amount of LPS is >1,000-fold less than that required by Rothstein and Schreiber to induce a minimal level of synergy (resulting in deaths) with 10 μg of rTNF. Thus, our data suggest the possibility that the two cytokines, rIL-1 and rTNF, might synergize to mediate toxicity similar to that observed following administration of high doses of LPS. These data also defined the experimental limits for subsequent experiments with respect to the range of usable cytokine dosage combinations; i.e., a range of 500 to 2,000 U of rIL-1 per mouse could be used in combination with 5 or 7.5 μg of rTNF to achieve lethality consistently below 10%.

Another toxic manifestation associated with injection of LPS is induction of weight loss, which is maximal at 48 to 72 h postinjection (27). In addition to the lethality observed in response to combinations of rIL-1 and rTNF (Fig. 1), weight loss was measured 3 days after injection of sublethal doses of rIL-1 and rTNF administered alone or in combination. At 3 days after injection, 1,000 or 2,000 U of rIL-1 resulted in ≤4.1% weight loss, and injection of rTNF (5 or 7.5 μg) resulted in ≤6.9% weight loss when compared with saline-injected controls (Fig. 2). However, in combination, rIL-1 plus rTNF synergized to induce weight losses in mice which ranged from 17.2 to 27.3%, similar to or exceeding that induced by a tolerance-inducing dose (25 μg) of LPS (18.6 ± 2.1%).

Effect of administration of rIL-1 or rTNF or both on day 0 on ability to respond to LPS on day 3. In early endotoxin tolerance models established previously in several laboratories (11, 31), 25 μg of LPS was found to induce a state of tolerance to subsequent injection with LPS 3 days later, as assessed by the decreased capacity to produce CSF 6 h after endotoxin challenge. Since a single nonlethal injection of LPS has been shown to induce both IL-1 and TNF shortly after administration, the capacity of these two cytokines to induce a state of early endotoxin tolerance was tested. Figure 3 illustrates the capacity of mice injected on day 0 to respond to LPS on day 3 by producing CSF. As reported previously, the ability of mice treated on day 0 with LPS

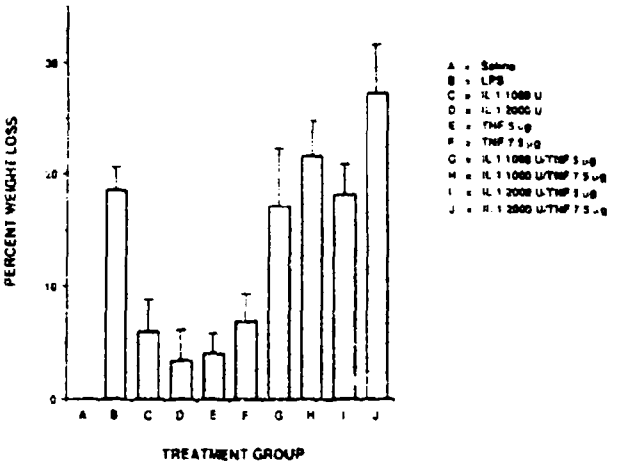

FIG. 2. Weight loss in response to injection with nonlethal doses of rIL-1 or rTNF or both. Mice were injected with saline, LPS (25 μg), or the indicated doses of rIL-1 or rTNF or both. Mice were weighed 3 days after injection, and the percent weight loss was calculated on the basis of the mean weight of saline-injected mice (17.2 ± 3 g). The data represent the arithmetic means ± the standard errors of the means of four separate experiments in which four to nine mice were injected per treatment group per experiment.

(treatment group B) to respond to LPS again on day 3 was <20% of the response of control mice (i.e., those which received saline on day 0; treatment group A). When mice were injected on day 0 with either rIL-1 or rTNF (treatment groups C to G), the ability to respond to LPS 3 days later was comparable to that of saline-pretreated mice. However, when mice were injected on day 0 with rIL-1 and rTNF in combination (treatment groups H to M), dose-dependent inhibition of LPS responsiveness on day 3 was observed. In addition, experiments in which doses as high as either 15,000 U of rIL-1 or 10 μg of rTNF were administered on day 0 led to no significant alteration in the ability to respond to LPS on day 3 (data not shown). These data indicate that in combined treatment of mice with rIL-1 and rTNF the two compounds synergize to mimic the tolerance-inducing effects of a sublethal dose of LPS.

Effect of administration of rIL-1 or rTNF or both on day 0 on bone marrow cell-sizing profiles and the number of macrophage progenitors on day 3. In previous studies, Madonna and co-workers (10–12) demonstrated that administration of a tolerance-inducing dose of LPS on day 0 led to a characteristic alteration in the cell-sizing profiles of bone marrow cells on day 3; i.e., there was enrichment for a population of larger mononuclear cells. Density gradient sedimentation studies showed that this larger population of cells contained increased numbers of progenitors which could respond to CSF-1 to form colonies in soft agar (11). Figure 4 shows a histogram analysis of the day 3 cell-sizing profiles of bone marrow cells derived from mice injected on day 0 with saline, LPS, rIL-1, rTNF, or rIL-1 plus rTNF. Figure 4A confirms the results of previous studies. When compared with saline injection of controls, injection of a tolerance-inducing dose of LPS on day 0 resulted in a marked shift in the cell-sizing profiles to a population of larger cells. Injection of either rIL-1 or rTNF resulted in a slight increase in the size of bone marrow cells (Fig. 4B; note the slight decrease in the proportion of cells in the 6.3- to 8.6-μm-diameter range and the compensatory increases within in the 8.7- to 11.4- and 11.5 to 14-μm-diameter ranges in mice treated with either rIL-1 or rTNF). These findings confirm and extend the results of a previous study in which rIL-1 was shown to increase bone marrow cell-sizing profiles (19). However, this IL-1-induced increase in population cell size fails to approach the magnitude of that observed in mice which received LPS on day 0 (Fig. 4A). Figures 4C and D show the effects of combined rIL-1 and rTNF treatment (on day 0) on the day 3 cell-sizing profiles. As observed after treatment with the tolerance-inducing dose of LPS (Fig. 4A), combined treatment with the two cytokines led to a marked shift in the cell-sizing profiles to a population enriched for significantly larger cells. In mice treated with both cytokines, there was enrichment for progenitor cells which respond to CSF-1 to form colonies in soft agar (Table 1). Thus, treatment of mice with both rIL-1 and rTNF resulted in the hematopoietic alterations previously reported to accompany a state of early endotoxin tolerance induced by LPS (11).

DISCUSSION

For many years, it has been recognized that the physiological changes that occur in experimental animals in response to gram-negative LPS are mediated principally by soluble factors produced by macrophages (reviewed by Vogel and Hogan [in press]). Perhaps the most convincing evidence for the participation of a particular cytokine in an LPS-induced response is demonstration of inhibition of a particular response by administration of anti-cytokine antibodies. To this end, the participation of IL-1 and TNF in

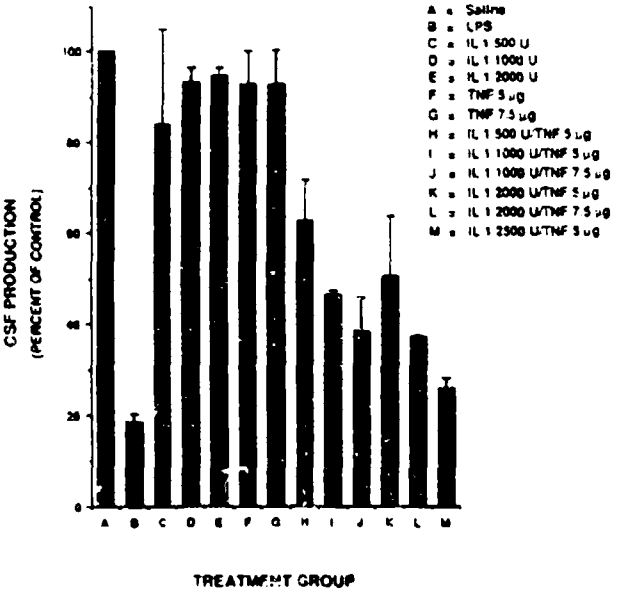

FIG. 3. Effect of injection of rIL-1 or rTNF or both on subsequent LPS responsiveness. Mice (four to seven mice per treatment group per experiment) were injected on day 0 with saline, LPS (25 μg), or the indicated doses of rIL-1 or rTNF or both. On day 3, mice were challenged with 25 μg of LPS and bled 6 h later. The pooled sera were subsequently tested for CSF activity (as described above). The data are expressed as percentages of the control (saline on day 0, LPS on day 3). The mean CSF activity of serum pools from mice which received saline on day 0 and LPS on day 3 was 6,237 ± 1,301 CFU/ml (five separate experiments). The results represent the arithmetic means ± the standard errors of the means for three to five separate experiments per treatment group.

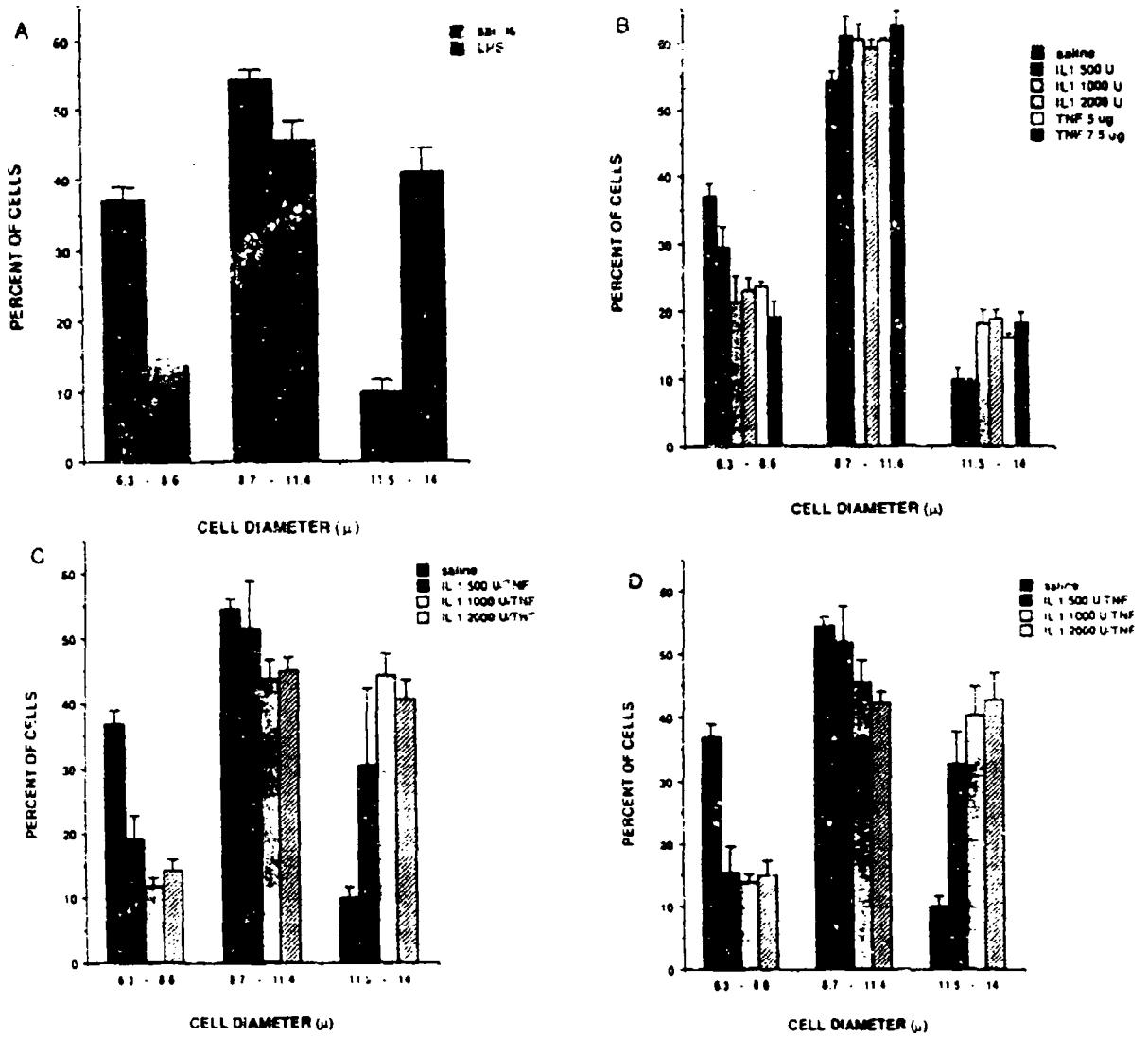

FIG. 4. Effect of rIL-1 or rTNF or both on cell-sizing profiles of bone marrow cells. Mice (two to three mice per treatment group per experiment) were injected on day 0 with saline, LPS (25 μg), or the indicated doses of rIL-1 or rTNF or both. On day 3, the bone marrow cells were obtained from the femurs and subjected to cell-sizing analysis with a Coulter Channelyzer as described in the text. Approximately 10,000 cells per treatment were analyzed for cell size in each experiment. The results represent the arithmetic means ± the standard errors of the means from three to seven separate experiments per treatment group. Panels: A, cell-sizing profiles from mice injected with saline versus LPS; B, saline versus various doses of either rIL-1 or rTNF; C, saline versus various doses of rIL-1 in combination with 5 μg of rTNF; D, saline versus various doses of rIL-1 in combination with 7.5 μg of rTNF.

LPS-mediated responses (e.g., fever and lethality) has been firmly established (4, 8). However, the availability of reagents such as anti-murine IL-1 and anti-murine TNF antibodies has been markedly limited, particularly for use in in vivo studies. It is also important to recognize that this experimental approach, although definitive for the participation of a particular factor, cannot preclude the possibility that the factor under study acts in combination with other coordinately induced mediators.

Another major approach which has been taken to assess the contribution of specific soluble factors in the mediation of LPS-induced effects is to test specific cytokines for their capacity to mimic LPS-induced responses. The recent availability of purified recombinant cytokines, such as rIL-1 and rTNF, has allowed for such an assessment without the earlier concerns which plagued studies in which natural cytokines were tested (i.e., the quantity of cytokine required for in vivo studies, as well as the persistent possibility that contaminating cytokines in the purified natural preparations actually induced the observed effect or in some way modified the response to the cytokine under question). Most recent studies using cloned reagents have focused on the administration of a single cytokine to induce a particular effect in vivo. In this regard, a plethora of information has come forth which indicates that, in vivo, both rIL-1 and rTNF induce many of the same LPS-like manifestations (reviewed in reference 6 and by Vogel and Hogan [in press]), even though these two factors bear no structural homology and bind to distinct receptors (2). For instance, both IL-1 and TNF have been shown to induce fever independently, via production of

TABLE 1. Effect of rIL-1 or rTNF or both on the numbers of CSF-1-responsive progenitors in bone marrow[a]

Treatment (amt)	Mean (± SEM) no. of CSF-1 progenitors/10^5 bone marrow cells (*P* value)[b]
Saline	47 ± 1.5
rIL-1 (1,000 U)	37 ± 10.8 (0.334)
rTNF (5 μg)	52 ± 7.8 (0.754)
rTNF (7.5 μg)	67 ± 3.5 (0.010)
rIL-1 (1,000 U)–rTNF (5 μg)	89 ± 5 (0.002)
rIL-1 (1,000 U)–rTNF (7.5 μg)	121 ± 1.9 (0)

[a] Bone marrow cells were derived on day 3 from mice (two to three mice per treatment group per experiment) which had received saline or the indicated dose of rIL-1 or rTNF or both on day 0. The results are expressed as the number of CSF-1-responsive progenitors per 10^5 bone marrow cells. The results represent three separate experiments.

[b] An unpaired, two-tailed Student *t* test was used.

prostaglandins (8). In this regard, they are both classical "endogenous pyrogens." In addition, at high doses, rTNF was shown to induce IL-1 in vivo (8). This particular example provides a good illustration of both cytokine redundancy (in the sense that two distinct cytokines can induce the same biological effect) and the potential for an inductive cascade which, in turn, could prolong a given manifestation in vivo. Injection of either rIL-1 or rTNF has been demonstrated in vivo to induce many of the same physiologic alterations seen in response to LPS in addition to fever, such as hypoglycemia, shock and death, increased resistance to infection, radioprotection, resistance to neoplasia, induction of CSF and late acute-phase reactants, and others (reviewed by Vogel and Hogan [in press]).

In this study, we sought to determine whether induction of early endotoxin tolerance by LPS is mediated by soluble factors produced in response to the tolerance-inducing injection (i.e., the sublethal injection of LPS given on day 0 to induce a state of resistance to a subsequent challenge 3 to 4 days later). In these studies, it was shown that neither rIL-1 nor rTNF injected individually (at doses which induced levels of circulating CSF in vivo comparable to that induced by a tolerance-inducing injection of LPS) induced a state of early endotoxin tolerance. This was assessed by production of normal levels of CSF in response to LPS administered 3 days later. Since IL-1 and TNF are coordinately produced in response to LPS, we hypothesized that induction of tolerance depends upon the simultaneous presence of both soluble factors. When administered individually, rIL-1 and rTNF rarely induced overt signs of toxicity; however, simultaneous administration of high doses of the two cytokines resulted in frank toxicity and often death. It is highly unlikely that this is due to synergy between rTNF and contaminating LPS, since the maximum amount of contaminating LPS injected was >1,000-fold less than that shown previously to induce minimal synergy with rTNF, resulting in death (22). Synergy between rTNF and rIL-1 was evident, even within a combined-dose range which rarely led to deaths, as measured by induction of weight loss greater than or equivalent to that induced by 25 μg of LPS. Within this same sublethal-dose range, the two cytokines induced refractoriness to LPS challenge in a dose-dependent fashion (Fig. 3). The amount of contaminating LPS injected with a more typical tolerance-inducing dose combination (e.g., 7.5 μg of rTNF and 2,000 U rIL-1) was <0.003 ng. Thus, it is more likely that the synergistic toxicity observed by Rothstein and Schreiber (22) is due to LPS induction of IL-1, which in turn synergizes with rTNF. These tolerance-inducing combinations of rIL-1 and rTNF also led to the hematopoietic alterations shown previously to be associated with LPS-induced early endotoxin tolerance (11), i.e., an increase in the size of bone marrow cells (Fig. 4) with concurrent enrichment for macrophage precursors (Table 1). With respect to the latter, it is interesting that injection of 7.5 μg of rTNF alone, but not 5 μg of rTNF, led to a statistically significant increase in the number of macrophage progenitors in the bone marrow. This may reflect the fact that high doses of TNF administered in vivo have been shown to induce IL-1 production (8). Thus, rTNF-induced IL-1 may act synergistically with administered rTNF to increase the number of CSF-1-responsive progenitors.

Taken collectively, these findings suggest that, in vivo, LPS-induced early endotoxin tolerance is mediated by the synergistic action of LPS-induced IL-1 and TNF. For most of the studies performed, combined administration of IL-1 and TNF has been shown to result in an additive effect; however, a precedent for synergy between these two factors exists. For example, Bevilacqua et al. (1) showed that intradermal injection of natural IL-1, like LPS, led to attraction of ^{51}Cr-labeled neutrophils to the site of injection. Movat et al. (17) confirmed these findings by using rIL-1 and extended them by demonstrating that the combined action of rIL-1 and rTNF was synergistic in producing infiltration. Similar findings were recently reported by Wankowicz et al. (30). Movat et al. (16) also demonstrated that intradermal injection of rIL-1 and rTNF led to synergistic induction of a local Schwartzman reaction in rabbits challenged intravenously with endotoxin 18 h later. Neta et al. (18) have shown that at doses of radiation which closely approach 100% lethality, the radioprotective effects of rIL-1 and rTNF are additive; however, at higher levels of irradiation, the radioprotection afforded by combined rIL-1 and rTNF injection was much greater than would be predicted by summing the protection afforded by injection of the cytokines individually. For induction of certain acute-phase reactants, such as serum amyloid P, simultaneous administration of rIL-1 and rTNF resulted in an additive response; however, combined administration of these two cytokines led to synergistic induction of fibrinogen (15). Recently, it was pointed out that IL-1 and TNF are strongly synergistic for generation of hypotension and the capillary leak syndrome (6, 20). In studies performed with C3H/HeJ mice, synergistic protection from infection by *E. coli* was afforded by treatment of mice with a combined rIL-1 and rTNF regimen (G. Saydoff, personal communication).

The mechanisms by which LPS induces a transient reversal of sensitivity to homologous or heterologous challenge with LPS, i.e., early endotoxin tolerance, are not very well understood. Early studies in which peritoneal macrophages or Kupffer cells of animals rendered tolerant to endotoxin were found to be refractory to stimulation with LPS in vitro (7, 21) strongly suggested that tolerance was a function of a failure to produce those soluble macrophage factors, such as endogenous pyrogen or prostaglandins, shown previously to be associated with endotoxin-mediated toxicity. Subsequent studies by Williams et al. (31) strengthened the role of macrophages in the induction of tolerance by showing that injection of either a splenic adherent cell population or peritoneal exudate macrophages along with splenic nonadherent cells was essential for overriding the inability to produce CSF in recipients rendered tolerant to endotoxin. Lastly, in work by Madonna and co-workers (10–12), the tolerance-inducing dose of LPS was followed by normal induction of macrophage-derived products, such as CSF and

interferon, but resulted in a depressed capacity to produce these same factors upon LPS challenge 3 to 4 days later. The kinetics of tolerance induction was correlated with acquisition of a characteristic change in the cell-sizing pattern of the bone marrow towards larger cells, and within this increased population of larger cells a marked increase in the number of CSF-1-responsive macrophage progenitors was observed. It was therefore proposed that (i) early endotoxin tolerance results from a developmental blockade which results in accumulation of immature macrophages in the bone marrow which, in turn, limits the number of fully mature, LPS-responsive macrophages in the periphery or (ii) initial exposure of mature macrophages to LPS renders them refractory to subsequent stimulation because of a blockade or down regulation of the LPS receptor.

The data presented in this report, that rIL-1 and rTNF synergize to induce refractoriness to LPS, as well as the hematopoietic changes reported previously, suggest that tolerance is induced in the absence of LPS and is mediated indirectly, rather than by a mechanism which involves a blockade of the LPS receptor. Another possibility which might be invoked to explain the failure to respond to a challenge injection of LPS is that the initial injection of the two cytokines results in induction of a state of tachyphylaxis to IL-1 or TNF produced in response to the LPS challenge. If this were so, then one would expect to observe diminished toxicity in the face of normal levels of circulating IL-1 or TNF produced in response to the LPS challenge. Preliminary experiments have indicated that, similar to the reduced levels of CSF and interferon in circulation following LPS challenge, animals rendered endotoxin tolerant produced significantly less circulating TNF upon challenge than did control animals (data not shown), consistent with earlier findings (7, 21) that macrophages derived from animals rendered tolerant produce depressed levels of soluble factors in vitro (e.g., endogenous pyrogen and prostaglandins) when exposed to LPS. Thus, failure to produce adequate levels of cytokines is more likely to underlie the observed decrease in toxicity, rather than cytokine-induced tachyphylaxis to TNF. Confirmation of these conclusions will depend upon the availability of high-titered anti-murine IL-1 and anti-murine TNF antibodies and the demonstration that injection of either reagent abrogates induction of LPS-induced tolerance in vivo. As indicated above, the hematopoietic changes observed in response to a tolerance-inducing dose of LPS are only a correlate of early LPS-induced tolerance. The demonstration that anti-IL-1 or anti-TNF or both antibodies also abrogate the hematopoietic responses to a tolerance-inducing dose of LPS would strengthen this relationship.

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